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Innate immune responses support adaptive immunity: NKT cells induce B cell activation

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Abstract

Invariant NKT cells are a peculiar subset of T lymphocytes whose features, highly conserved both in the mouse and the human system, strongly recall those of other "innate lymphocytes". Following recognition of CD1d-presented glycosphingolipid antigens invariant NKT promptly release high amount of diverse cytokines concurring to the activation of the actors of both innate and acquired immune responses. For this reason, in recent years NKT cells have been the object of intensive study, aimed to understand their role in diverse patho-physiological conditions and to exploit the possibility to take advantage of their "adjuvant-like" activity in the formulation of new vaccines. As antibodies are an essential part of many immune responses, we focused our attention on invariant NKT–B cell interactions analyzing their influences on B cell activation and effector functions. The results of this study demonstrate that human invariant NKT cells can provide direct help for B cell proliferation and antibody production through CD1d-restricted mechanisms. Remarkably, help to B lymphocytes by invariant NKT cells is delivered also in the absence of exogenous antigen, suggesting the existence of an endogenous ligand presented by CD1d on B cells.

Keywords: α-Galactosylceramide; NKT; Helper activity

1. Introduction

NKT cells are a heterogeneous subset of T lymphocytes characterized by the co-expression of a TCR and the natural killer receptor NK1.1/NKRP1A (CD161). Of at least four groups of NKT cells which could be identified so far [1,2], the best characterized one displays a $CD4^+$ or $CD4^-CD8^-$ double negative (DN) phenotype and expresses a semi-invariant TCR, encoded in mice and humans by the homologue invariant Vα14–Jα281 and Vα24–JαQ rearrangements, respectively [1–6]. The two invariant TCR Vβ chains pair with a restricted TCR Vβ repertoire, comprising Vβ8, Vβ7 or Vβ2 chains in mice [5,6] and Vβ11 in humans [7–9]. Because of their peculiar TCR repertoire, these cells are referred to as invariant NKT cells. Differently from conventional T lymphocytes, invariant NKT cells have a prominent activated/effector phenotype and the ability to promptly release high amounts of IFN-γ and IL-4 upon primary TCR stimulation [10–12]. For these reasons invariant NKT cells are regarded as actors of the innate immune response [10], playing a major adjuvant-like role during an immune response [2,5], spanning from NK cell activation [13], helper T cell differentiation [14], to the control of autoimmune diseases [15,16], infections [17] and tumor growth [18].

Both mouse and human invariant NKT cells recognize the highly conserved, MHC class I-like molecule, CD1d [19–21]. Although the identification of their natural antigen(s) is still elusive, both mouse and human invariant NKT cells potently react to α-galactosylceramide (α-GalCer), a glycosphingolipid isolated from marine sponges that specifically binds CD1d [22–24]. CD1d molecules are expressed on cells both of hematopoietic and non-hematopoietic origin [21]. Intriguingly, the majority of mouse and human B cells expresses CD1d, suggesting that direct interactions between invariant NKT and B lymphocytes can, theoretically, occur and affect B cell effector functions. Data from mouse models of immunopathology or infection, indeed suggest that the interaction between CD1d expressed on B cells and CD1d-restricted T cells may influence the amount, isotype and specificity of the produced antibodies [5,6,17,25]. Nevertheless, attempts to demonstrate specifically that these effects are consequence of direct, CD1d-dependent, invariant

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NKT–B cell interactions have produced conflicting results in mice [26,27] while nothing is known in humans.

To directly address this question we tested the ability of human invariant NKT cells to activate autologous purified B lymphocytes in vitro, and provide them help to proliferate and display effector functions. The results of this study demonstrate that human invariant NKT cells can provide direct help for B cell proliferation and antibody production through CD1d-restricted mechanisms. Ability to help B cells is shared by both $CD4^+$ and DN NKT cells, although the two subsets display significant differences in supporting immunoglobulin production. Remarkably, help to B lymphocytes by invariant NKT cells is delivered also in the absence of exogenous antigen, suggesting the existence of an endogenous ligand presented by CD1d on B cells.

2. Materials and methods

2.1. Antibodies and flow cytometric analysis

Specific monoclonal antibodies used for this study were: FITC, PE, PerCP, APC or biotin-conjugated anti-Vβ11 (IgG2a, clone C21), anti-Vα24 (IgG1, clone C15) (Immunotech, Marseille, France); anti-CD1d (IgG1, clone CD1d42), anti-CD3 (IgG1, clone SK7), anti-CD4 (IgG1, clone RPA-T4), anti-CD8 (IgG1, clone SK1), anti-CD14 (IgG2a, clone M5E2), anti-CD20 (IgG1, clone L27), anti-CD27 (IgG1, clone L128), anti-CD161 (IgG1, clone BX12), anti-IFN-γ (IgG2b, clone 25723.11), anti-IL-4 (IgG1, clone 3010.211), anti-IL-13 (IgG1, clone JES10-5A2), anti-TNF-α (IgG1, clone 6401.1111), IgG1 and IgG2a isotype control antibodies (all from BD Biosciences).

Antibodies used in cell cultures were: anti-CD3 (IgG1, clone UCHT1), anti-CD1d (IgG1, clone CD1d42), anti-CD4 (IgG1, clone RPA-T4), anti-IL-4 (rat IgG1, clone MP425D2), anti-IL-13 (rat IgG1, clone JES105A2); all from BD Biosciences, agonistic anti-CD40 (IgG1, purified from supernatant of the 626.1 hybridoma cell line); mouse IgG1 fraction was purchased from Sigma.

The expression of cell surface molecules and intracellular cytokines was analyzed by four-color flow cytometry using a LSR® flow cytometer (Becton Dickinson) following standard protocols [28]. For all samples, at least 10^4 events in the lymphocyte region were acquired. Results were analyzed using the CellQuest® software (Becton Dickinson).

2.2. Derivation of invariant NKT and T cell clones

Human PBMC were obtained from heparinized peripheral blood of three healthy volunteers after density gradient centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden). All cultures were maintained in RPMI medium (Gibco) containing 10% FCS (HyClone), 2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate and antibiotics (Gibco), hereafter referred to as complete medium. To expand α-GalCer responsive T cells, PBMC were plated at 10^6 cells/ml in 24-well plates (Corning Costar) containing 2 ml per well of complete medium and 50 ng/ml of α-GalCer (KRN7000; Kirin Brewery Co. Ltd., Gumna, Japan; supplied to P. Dellabona). One hundred units per ml of human recombinant IL-2 (Chiron, Emeryville, CA, US) were added after 24 h. At day 15, αGalCer-induced cell lines were analyzed for Vβ11, Vα24, CD8 and CD4 expression by four-color flow cytometry and sorted with a FACSVantage-SE® flow cytometer using the CellQuest® software (Becton Dickinson). $Vα24^+Vβ11^+CD4^+CD8^-$, $Vα24^+Vβ11^+CD4^-CD8^-$ and $Vα24^-Vβ11^-CD4^+CD8^-$ T cells were cloned using the CloneCyte Plus® software at 1 cell per well, in 60-well Terasaki plates (Robbins, Sunnyvale, CA, US) containing 40 μl of irradiated (6000 rad) PBMC as feeder cells (2×10^6 cells/ml), PHA (0.5 μg/ml, Sigma) and recombinant human IL-2 (100 U/ml). Invariant NKT and T cell clones were then maintained in complete medium and re-stimulated with fresh irradiated feeder cells, PHA and IL-2 every 15–20 days. To assess their specificity for α-GalCer, NKT or T cells were plated at 2.5×10^4 to 5×10^4 T cells per well in U-bottom 96-well plates (Corning Costar) together with 5×10^4 irradiated (6000 rad) antigen-presenting cells (APC), in the presence of 0.2 μg/ml anti-CD3 or 50 ng/ml of α-GalCer. Parallel cultures were run in the presence of 0.2 μg/ml of total mouse IgG1 or DMSO (a volume equivalent to that present in α-GalCer cultures), and considered as background. APC were $CD19^+$ cells, purified from the same individuals from which NKT cell clones were derived (see further). After 48 h, cell proliferation was assessed by [^{3}H]thymidine incorporation, by adding 1 μCi per well of [^{3}H]thymidine for additional 16 h. Cells were then harvested onto filter plates (Packard, Meridien, CT, US) and radioactivity was measured using a TopCount NXT® apparatus (Packard). Six $Vα24^-Vβ11^-CD4^+$ α-GalCer-unreactive T cell clones and 12 $Vα24^+Vβ11^+$ α-GalCer-reactive NKT cell clones (6 $CD4^+$ and 6 $CD4^-$) were expanded further to be used in this study. The presence of canonical Vα24–JαQ rearrangement in $Vα24^+Vβ11^+$ NKT cell clones was confirmed by PCR-heteroduplex analysis as previously described [11]. All 18 clones were routinely monitored for CD1d, CD3, CD4, CD8, Vα24, Vβ11 and CD161 expression.

2.3. B lymphocyte's purification and helper assays

Autologous $CD19^+$ cells were purified from peripheral blood of the same individuals from which NKT cell clones were derived. Briefly, PBMC were incubated with an anti-CD19 mAb conjugated with magnetic beads, and magnetically labeled cells were purified on LS+ columns using the MACS® separation system (Miltenyi). Positively selected fractions were assessed for CD1d, CD3, CD14, CD20, CD40 and CD56 expression. All B lymphocyte preparations were more than 99% $CD20^+$, the few contaminating cells being $CD14^+$.

To assess the helper function of NKT cell clones, 5×10^4 purified B lymphocytes were seeded alone or together with 2.5×10^4 irradiated (6000 rad) NKT cells, in U-bottom 96-well plates containing 0.2 ml of complete medium. Duplicate or triplicate wells were stimulated with 0.2 μg/ml anti-CD3, 50 ng/ml of α-GalCer or equal concentrations of mouse IgG1 or DMSO. After 5 days in culture, 1 μCi per well of [^{3}H]thymidine was added to each well for 16 h, before cell harvesting and scintillation counting as described above. Immunoglobulin concentration in supernatants collected on day 10 of culture was determined by sandwich ELISA following manufacturer's instructions (BD Biosciences). The monoclonal antibody pairs used were: anti-human IgM clone JDC-15 (capture) and biotin-conjugated clone G20-127 (detection); anti-human IgG1 clone G18-145 (capture) and biotin-conjugated clone G17-1 (detection); anti-human IgE clone G7-18 (capture) and biotin-conjugated clone G7-26 (detection). Total human IgG1, IgM (both from Sigma) and IgE (Calbiochem, Darmstadt, Germany) were used as standards.

2.4. CFDA-SE dilution assay

Purified B cells were loaded with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Molecular Probes, Leiden, The Netherlands) following standard protocols [29]. B lymphocytes were then seeded at 2×10^5 to 5×10^5/ml in U-bottom tubes containing complete medium, with or without 10 μg/ml anti-CD40 or 50 ng/ml α-GalCer, in the presence or in the absence of non-irradiated invariant NKT cells (in a ratio of 20 B cells to 1 NKT cell). Four color-cytofluorimetric analysis of CFDA-SE, PE-anti-CD27, PerCP-anti-CD3 and APC-anti-CD20 stained cells, was performed on $CD20^+CD3^-$ lymphocytes, using the CellQuest® software. The presence in this region of dead cells was excluded by running parallel propidium iodide-stained samples. For each sample, at least 2×10^5 events in the lymphocyte region were acquired.

2.5. Statistical analysis

Statistical analysis was performed using the paired Student's *t*-test.

3. Results

3.1. Human $CD4^+$ invariant NKT cell clones induce both naïve and memory B lymphocytes proliferation and effector functions, even in the absence of α-GalCer

PBMC of three healthy individuals were cultured with α-GalCer in vitro and a panel of invariant $CD4^+$, $V\alpha24^+$, $V\beta11^+$ T cell clones specific for this glycolipid was obtained. To assess whether $CD4^+$ invariant NKT cells were able to help B lymphocytes, autologous B cells were purified from peripheral blood and analyzed for the expression of the memory marker CD27 [30] and of the NKT cell restriction molecule CD1d. No significant differences in CD1d expression were observed between naïve ($CD27^-$) and memory ($CD27^+$) B cell subsets (data not shown).

B cells were then loaded with the fluorescent vital dye CFDA-SE and cultured in the presence of $CD4^+$ invariant NKT cell clones, with or without α-GalCer. As controls, purified B cells were also cultured in the presence of cytokines or an agonistic anti-CD40 mAb. After 5 days, B cell proliferation was determined, by assessing the dilution of the CFDA-SE dye in both the $CD27^+$ and $CD27^-$ B cell subsets. While no CFDA-SE dilution was observed in samples of purified B cells cultured with IL-2 plus IL-4 (indicating that B cells were not pre-activated; data not shown), those cultured with the agonistic anti-CD40 mAb showed detectable cell division, that was mainly restricted to the memory ($CD27^+$) cell subset (Fig. 1A). When B cells were co-cultured with invariant NKT cell clones, both memory ($CD27^+$) and naïve ($CD27^-$) B lymphocytes divided, as indicated by CFDA-SE dilution (Fig. 1A). Although maximal proliferation was observed in the presence of both invariant NKT cells and α-GalCer (Fig. 1A), significant B cell division was observed also when they were cultured in the presence of invariant NKT cells without α-GalCer (Fig. 1A). Both in the presence and in the absence of α-GalCer, the B cell proliferation induced by invariant $CD4^+$ NKT cells, was almost completely inhibited by a neutralizing anti-CD1d antibody.

From the above experiments we conclude that not only $CD4^+$ invariant NKT cells induce proliferation of both naïve and memory B cells, but most interestingly, that even in the absence of α-GalCer, invariant NKT cells help proliferation of autologous B cells in a CD1d-restricted manner.

Having demonstrated the ability of invariant NKT cells to induce B cell proliferation, we analyzed their ability to support immunoglobulin production. Irradiated $CD4^+$ invariant NKT cells were then cultured with autologous purified B cells, in the presence either of the polyclonal T cell stimulus anti-CD3 or of the invariant NKT-specific antigen α-GalCer. After 10 days, the presence of IgM, IgG1 and IgE in the culture supernatants was assessed. As shown in Fig. 1B and C, both IgM and IgG1 production were induced by $CD4^+$ invariant NKT cell clones activated by anti-CD3 or by α-GalCer, while IgE production was never observed under any circumstances (data not shown). When an anti-CD1d neutralizing antibody was added to invariant NKT–B cell cultures, the subsequent immunoglobulin production by B cells was inhibited when invariant NKT cells were activated by α-GalCer, but not when they were activated by anti-CD3 (Fig. 1B and C). Interestingly, $CD4^+$ invariant NKT cells helped low but significant levels of IgM (Fig. 1B), but not IgG1 (Fig. 1C), production by autologous B cells in the absence of any T cell activation stimuli. Also in this case, antibody production was inhibited by a neutralizing anti-CD1d antibody. These results further

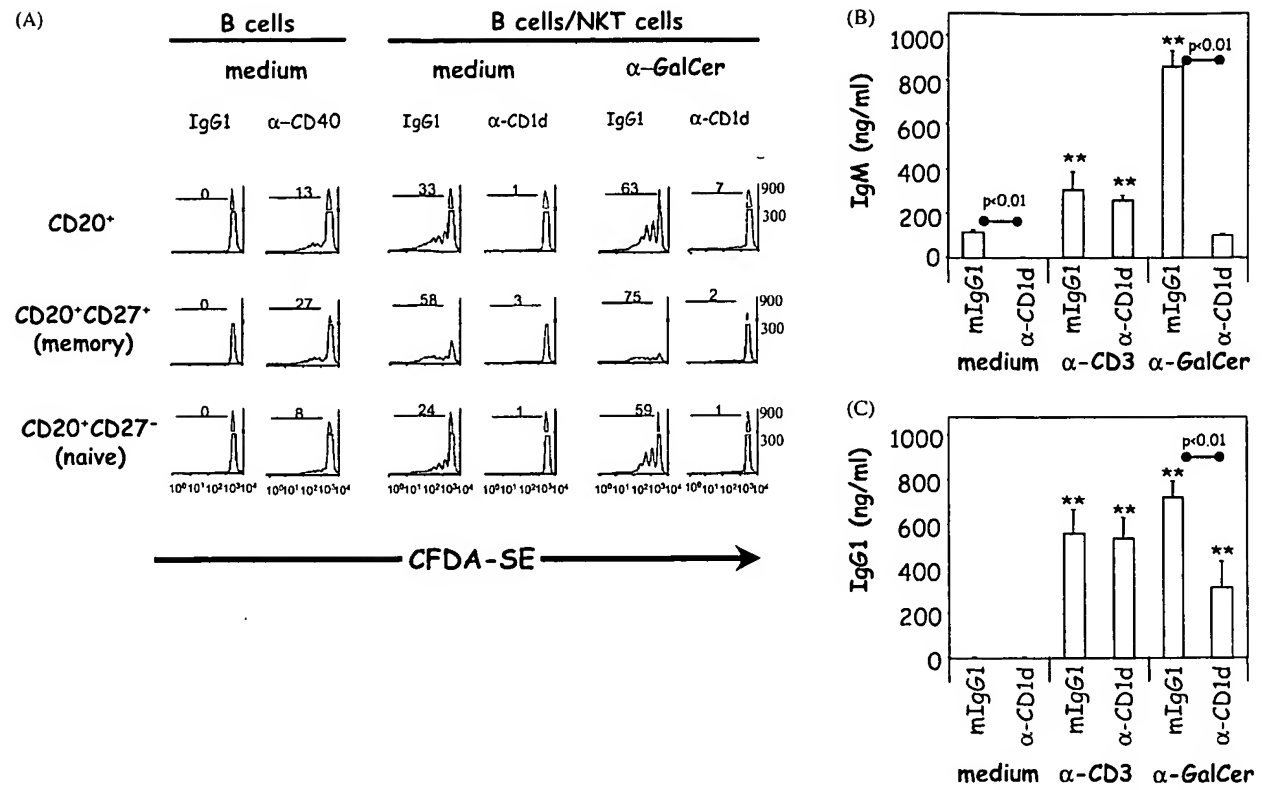

Fig. 1. Human $CD4^+$ invariant NKT cell clones induce CD1d-dependent proliferation of B lymphocytes and immunoglobulin production in the presence and in the absence of α-GalCer. (A) Flow cytometric analysis of CFDA-SE dilution on $CD27^+$ and $CD27^-$ B lymphocytes after 5 days in culture in complete medium with or without an agonistic anti-CD40 mAb or a $CD4^+$ invariant NKT cell clone in the presence or in the absence of α-GalCer, neutralizing anti-CD1d or isotype control mAbs. Results are from one representative experiment of three, in which three different $CD4^+$ NKT cell clones were tested in parallel giving comparable results. (B) IgM and (C) IgG1 released in 10 days supernatants by B lymphocytes stimulated by irradiated $CD4^+$ invariant NKT cell clones and medium, anti-CD3 or α-GalCer, in the presence of a neutralizing anti-CD1d or isotype control mAbs. Results show the mean ± S.E. of values from three independent experiments, each performed with three $CD4^+$ invariant NKT cell clones. *P*-values between anti-CD1d and isotype control mAb-treated samples are indicated above the lines. ** Indicate $P < 0.01$ against medium.

demonstrated that human invariant NKT cells, probably recognizing a yet unidentified ligand, can help autologous B cells in the absence of α-GalCer.

3.2. *Human $CD4^+$ and DN invariant NKT cells differ in their ability to support immunoglobulin production, but provide similar help for B cell proliferation*

There exist two major subsets of invariant NKT cells: $CD4^+$ and DN (double negative $CD4^-CD8^-$). We assessed whether $CD4^+$ and DN NKT cell subsets displayed different helper activity in respect with B cell proliferation and immunoglobulin production. Since these two subsets have been described to display different cytokine profiles, with the DN one producing higher levels of Th1 than Th2 cytokines [2–4], we first compared phenotype and cytokine production of our $CD4^+$ and DN invariant NKT cell clones with those of conventional $CD4^+$ Th0 cell clones obtained from the same individuals, taken as paradigm for helper activity. Differently from what described for ex vivo purified invariant NKT cells [2–4], PMA and ionomycin activated $CD4^+$ and DN invariant NKT cell clones expressed levels of CD40L (Fig. 2A) and pattern and levels of cytokines (Fig. 2B) comparable to those of conventional $CD4^+$ Th0 cell clones. Consistent with this finding, the two subsets of invariant NKT cells, when activated by anti-CD3 or α-GalCer, induced similar levels of B cell proliferation (Fig. 2C). By contrast, however, $CD4^+$ invariant NKT cells were significantly more efficient than the DN ones in eliciting IgM and IgG1 production by B cells (Fig. 2D and E). We conclude that $CD4^+$ and DN NKT cells differ in their ability to support immunoglobulin production in the face of comparable CD40L expression and cytokine production.

4. Discussion

The present study demonstrates that human invariant NKT cells are able to provide direct help to autologous B lymphocytes, inducing proliferation of both naïve and memory B cells as well as IgM and IgG production.

Productive interaction between invariant NKT cells and B lymphocytes requires CD1d molecules on B cell surface.

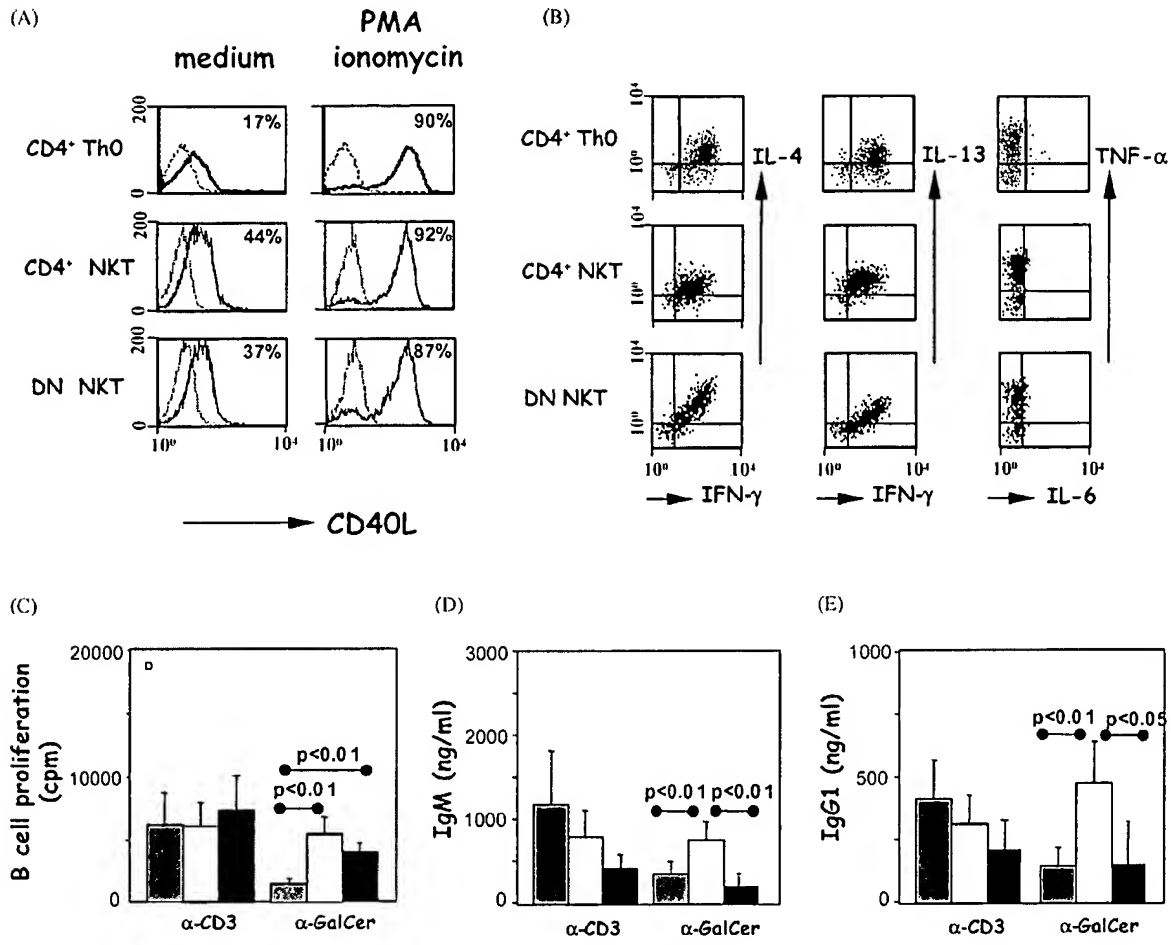

Fig. 2. Both human $CD4^+$ and DN invariant NKT cell clones help B cell proliferation, but $CD4^+$ NKT cell clones sustain higher immunoglobulin production. The expression of CD40L (A) and of several intracellular cytokines (B) was measured on conventional $CD4^+$ Th0 cell clones, $CD4^+$ and DN invariant NKT cell clones. Results are from one clone representative of six for each group. B lymphocyte proliferation (C) IgM (D) and IgG1 (E) immunoglobulin production following stimulation by irradiated conventional $CD4^+$ Th0 cells (gray bars), $CD4^+$ (open bars) or DN (black bars) invariant NKT cell clones in the presence of anti-CD3 or of α-GalCer (basal levels have been subtracted). Results are the mean ± S.E. of values from three independent experiments in which six clones for each group were tested. *P*-values between samples activated with the three groups of clones are indicated.

Notably, invariant NKT cells are reactive to CD1d expressed on primary B lymphocytes even in the absence of α-GalCer. Although we cannot formally rule out carry over of α-GalCer used in the original stimulation of peripheral blood lymphocytes from which $V\alpha24^+V\beta11^+$ T cell lines were expanded, usage of invariant NKT cell clones after at least 20 days of culture in the absence of any exogenous α-GalCer (see Section 2) make this possibility extremely unlikely. Furthermore, all the invariant NKT cell clones used in this study were always found CD1d negative. Therefore, we would like to put forward the hypothesis that B cells express an endogenous glycolipid, other than α-GalCer, associated to CD1d and that this is sufficient to activate NKT cells.

Strictly related to the identity of this putative endogenous antigen is the key to understand the functional relevance of the invariant NKT cell help. In the classical model of MHC-II-dependent cognate help, BcR-mediated antigen internalization and presentation of specific epitopes to antigen-specific T cells is required [31]. Is this in vitro interaction between B and invariant NKT cells restricted in vivo to those B lymphocytes endowed with BcR specific for glycosphingolipid antigens, that can thus be captured, internalized and presented in the CD1d context to invariant NKT cells?

Alternatively, it may be that invariant NKT cells recognize those B cells that express a yet unknown endogenous ligand associated to CD1d, regardless of their BcR specificity. This possibility would require tight control to avoid generalized B cell activation. For instance, invariant NKT cells might help B cells in which CD1d presents endogenous ligands that are synthesized by B cells upon stimuli such as inflammatory/innate signals via Toll-like receptors [32].

Indeed, it has been recently described [33] that only a minority (about 4%) of circulating $CD4^+$ invariant NKT expresses the specific set of homing receptors required to circulate within secondary lymphoid organs [34,35], while

virtually no $CCR7^+CD62L^+$ cell was found in the DN invariant NKT cell subset [33]. These observations strikingly parallel our findings that the $CD4^+$ invariant NKT cell subset provides a more efficient help for immunoglobulin production than the DN one, despite the two subsets exert comparable helper activity toward B cell proliferation. Therefore, it is tempting to speculate that, under physiological conditions, intranodal interactions with CD1d-expressing B lymphocytes are restricted only to those $CD4^+$ invariant NKT endowed with appropriate lymphoid tissue-homing receptors.

On the other hand, given that the great majority of invariant NKT cells is equipped with homing receptors for inflamed tissues, such as CXCR3 and CCR5 [33], also extranodal interactions between invariant NKT cells and B cells are likely to occur at inflamed non-lymphoid tissues. Ectopic lymphoid follicles containing activated B cells are often found in tissues chronically inflamed because of autoimmune diseases [36] or viral infections [37]. Furthermore, lymphoid aggregates detected in the liver peri-portal spaces of patients with chronic hepatitis C, contain high numbers of B cells expressing CD1d (our unpublished observations). It may thus well be that invariant NKT cells contribute to the local immune response when activated by B cells that have migrated into inflammation sites, even if the inflamed tissue is CD1d negative. Consequently, the CD1d dependent interaction between invariant NKT and B cells would also result in activation of B cells to proliferate and/or to produce immunoglobulins.

While invariant NKT cells produce both IFN-γ and IL-4, it is not clear whether their effector functions influence adaptive immune responses toward Type 1 or Type 2 responses. In our experimental system, invariant NKT cells, like conventional $CD4^+$ Th0 cells, did not induce detectable IgE production (the hallmark of a Th2 response). This finding is in agreement with the reduction of IgE levels observed in sera of mice injected with α-GalCer [38], but it is different from other studies showing that repeated inoculation of α-GalCer in mice promote an increase in IgE production in the context of a global Th2-switch of the immune response [14,39]. These apparent discrepancies probably reflect differences in the various experimental settings that could favor co-stimulatory pathways required by invariant NKT cells to preferentially produce Th1 or Th2 cytokines [40].

The role played by NKT cells in the regulation of the B cell responses should be now investigated in depth in different models, especially in view of our finding that NKT cells help B cells in the absence of α-GalCer, suggesting a regulatory role of NKT cells on B cell physiology.

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Natural killer T cells as targets for therapeutic intervention in autoimmune diseases.

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Abstract

Natural killer T (NKT) cells are a subset of lymphocytes that express receptors characteristic of conventional T cells together with receptors typically found on natural killer cells. A key feature of NKT cells is the expression of a semi-invariant T cell receptor that is specific for glycolipid antigens presented by the unusual major histocompatibility complex class I-like molecule CD1d. While their precise immunological functions remain unknown, NKT cells have been implicated in the regulation of adaptive immune responses, including those directed against autoantigens. These findings raise the possibility that specific stimulation of NKT cells may be exploited for therapeutic purposes. A number of laboratories have tested this hypothesis, utilizing the sea sponge-derived agent alpha-galactosylceramide (alpha-GalCer), a specific agonist of NKT cells. Administration of alpha-GalCer to mice results in potent activation of NKT cells, rapid and robust cytokine production, and activation of a variety of cells of the innate and adaptive immune systems. Most notably, repeated administration of alpha-GalCer to mice favors the generation of conventional T lymphocytes producing T helper (Th) type 2 cytokines such as IL-4 and IL-10. These findings suggest that alpha-GalCer can modulate inflammatory conditions that are mediated by pathogenic Th1 cells. Indeed, recent studies have demonstrated that alpha-GalCer prevents the development of Type 1 diabetes in non-obese diabetic mice and central nervous system inflammation in mouse models of multiple sclerosis. Collectively, these studies provide a solid foundation for the development of NKT cell ligands as pharmacological agents for treatment of autoimmune diseases.

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